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Molecular evidence for the occurrence of *Tomato leaf curl New Delhi virus* on bottle gourd in Tamil Nadu, India

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Abstract

Bottle gourd plants showing disease symptoms of mosaic mottling, chlorosis and yellowing of leaves from two different locations of Tamil Nadu were found to be infected with a *Begomovirus* through PCR assay with universal primer pair (Deng 540/541). In BLAST analysis, it is identified as *Tomato leaf curl New Delhi virus* (ToLCNDV). Additionally amplified complete coat protein gene using another primer pair GK ToLCV F/R were sequenced and deposited to the GenBank database (TN MET BoG2 - KM275606; TN NGK BoG1 - KM275616). The nucleotide of TN MET BoG2 and TN NGK BoG1 had maximum identity of 94 % and 98% towards the ToLCNDV reported from Spain and Asian countries respectively. In the phylogenetic analysis, TN NGK BoG1 clustered with ToLCNDV isolate infecting *Parthenium* whereas TN MET BoG2 clustered with isolates infecting cucrbitaceous crops from Spain. As per our knowledge this is the first confirmed molecular evidence for the occurrence of *Tomato leaf curl New Delhi virus* on bottle gourd in Tamil Nadu.

Keywords: Cucurbits virus, Mosaic disease, PCR, Bottle gourd, Cucurbits

Introduction

Bottle gourd (*Lagenaria siceraria*) belongs to Cucurbitaceae is one of the important vegetable crop grown in Tamil Nadu. Despite having a rich source of Vitamin A and Vitamin C, it is also having many medicinal properties (Robinson and Deckers-Walters, 1997). Viruses reported to cause threat on bottle gourd cultivation globally are *Cucumovirus* (Takeshita *et al*., 2001), *Potyvirus* (Mantri *et al*., 2004; Verma *et al*., 2004), *Begomovirus* (Sohrab *et al*., 2010), etc. From past two decades whitefly transmitting begomoviruses were causing major threat to cucurbitaceous crops in addition to their occurrence on solanaceous vegetables. In cucurbits, begomoviruses reported in India were *Tomato leaf curl New Delhi virus* (ToLCNDV) (Nagendran et al., 2014a) and *Squash leaf curl china virus* (SLCCNV) (Nagendran et al., 2014b; Saritha et al., 2011). ToLCNDV was reported not only on cultivated crops but also on many weed plants associated with the cropping ecosystem such as *Eclipta* and *Croton* (Mandal, 2010). In the survey on mosaic disease of cucurbits conducted during 2012-14, an outbreak of virus like disease was occurred on bottle gourd in the farmer's field at Mettur and Nagercoil regions of Tamil Nadu under natural conditions. Hence, an attempt was made to identify the virus associated with this disease on bottle gourd in Tamil Nadu.

Materials and Methods

Survey and Collection of plant samples: Surveys were conducted during 2012-2014 in major cucurbit growing regions of Tamil Nadu to study the virus diseases. During our survey, bottle gourd samples from Mettur and Nagercoil regions of Tamil Nadu showing mosaic and yellowing were collected along with healthy plant samples and brought to laboratory for molecular analysis of the associated virus with the symptoms. The samples were stored at -80^{o}C till the further identification studies. Disease incidences were estimated by recording symptomatic and non-symptomatic plants in the field randomly at ten different locations with one square meter each (Sohrab *et al*. 2010).

Total DNA extraction and PCR amplification: The total DNA was extracted from 100 mg leaves tissue of infected as well as healthy leaf samples using CTAB

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method described by Doyle and Doyle (1990). The genomic DNA quality and quantity was checked on 0.8% agarose gel and stored at -20°C till further use. For preliminary screening, PCR amplification has been carried with *Begomovirus* specific degenerate primer pair of Deng 541: 5' TAATATTACCKG WKGVCCSC 3' Deng 540: 5' TGGACYTTRCAWGGBCCTTCACA 3' to amplify a part of the movement protein and coat protein (CP) genes of DNA-A (Deng *et al*., 1994). To amplify the complete coat gene, another primer pair (GKToLCV F: ATGKYGAAGCGACCAGCMGA; GKToLCV R: CGCCCKCMGAYTGGGMTTTTCTT) were used (Nagendran et al. 2014a). Amplified products were cloned, sequenced and submitted to the NCBI database.

Sequence comparison and Phylogenetic analysis: The sequences of this study isolates were compared with the selected begomovirus sequences from the GenBank database. Multiple sequence alignment was done using ClustalW followed by phylogenetic analysis using MEGA 6.0 and tree was constructed with the neighbor-joining algorithm, bootstrapped with 1000 replicates (Tamura et al. 2013). Also similarity matrix was generated using BIO-EDIT (Hall 1999).

Results

Field symptoms, disease incidence and detection of *Begomovirus*: During survey on virus diseases of cucurbits in Tamil Nadu from 2012 to 2014, mosaic disease was observed on bottle gourd fields in Mettur (Salem) and Nagecoil. In Mettur, symptoms were mosaic mottling and chlorotic spots on leaves with a percent disease incidence of 45.4% and in Nagercoil, symptoms appeared as chlorosis and yellowing of leaves with a percent disease incidence of 77.6%.

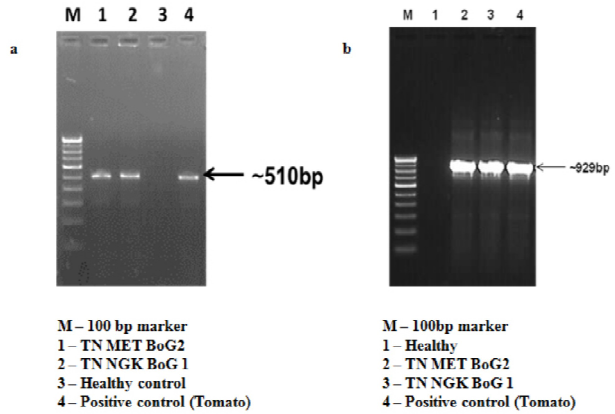

Fig. 1. Gel photograph showing amplification of ToLCNDV using universal Begomovirus primer pairs Deng 540/541 (a) and ToLCNDV specific primer pairs GK ToLCV F/R (b)

Detection and Characterization of virus: DNA extracted from the symptomatic and non-symptomatic leaves were subjected to PCR for preliminary screening of presence of *Begomovirus* using Deng 540/541 primer pairs. Symptomatic leaves showed amplification of ~510bp but not in non-symptomatic leaves (Fig. 1a) which confirms the presence of *Begomovirus* associated with the mosaic diseases of bottle gourd. For further confirmation, another set of primer pair (GKToLCV F/R) was used to amplify complete coat protein gene (771nt) of ToLCNDV and SLCCNV to a amplification size of 929 bp. Amplified product were cloned and sequenced (Fig. 1b).

Sequence analysis: BLASTn analysis of nucleotide sequence data of the complete coat protein gene of TN MET BoG 2 (KM275606) isolate revealed a maximum identity of 94% with Spain isolates of ToLCNDV infecting Cucurbits (KF749223, KF749223, KF891468) and Tomato infecting Gujarat isolate (KF551576). Similarly coat protein nucleotide sequence of TN NGK BoG 1 (KM275616) isolate shared a maximum identity of 98% with several isolates of ToLCNDV reported from India: Maharastra (HQ264185), Iran (KJ778692) and Pakistan (KF002409). Further nucleotide analysis of ToLCNDV-TN MET BoG2 shared maximum similarity of 97.2% at aminoacid level with the ToLCNDV isolates from Spain (KF749223, KF749223 and KF891468) and 96.4% similarity towards ToLCNDV isolate from India (KF551576). Also ToLCNDV – TN NGK BoG1 shared 99.6% similarity at aminoacid level with the various isolates from India (HQ264185), Iran (KJ778692) and Pakistan (HQ264185) (Table 1). Also both the isolates TN MET BoG2 and TN NGK BoG1 shares only 91% and 94% identity among themselves at nucleotide and aminoacid level respectively. In phylogenetic analysis, nucleotide sequence of TN MET BoG2 formed a separate cluster along with the ToLCNDV isolates infecting cucurbits from Spain and TN NGK BoG1 forms separate clade under ToLCNDV subgroup along with Asian isolates of ToLCNDV reported from India, Iran and Pakistan (Fig. 2).

Discussion: In the survey conducted on virus diseases of cucurbits in Tamil Nadu during 2012-14, bottle gourd plants on farmer's fields showed virus like symptoms in Mettur and Nagercoil region. In Mettur, plants expressed chlorotic mosaic patches on the leaves and in Nagercoil plants expressed entirely different types of symptoms *viz*., chlorosis and yellowing of leaves without any mosaic patches. Similarly, Abdalla and Ali (2013) reported that chlorotic spots, yellowing, mottling, vein clearing and mild mosaic were found to be associated with the virus infection in Squash. Saritha et

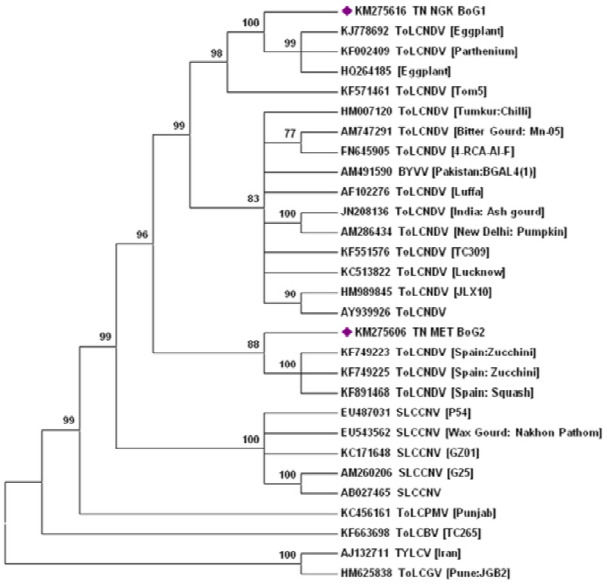

Fig. 2. Phylogeny of complete nucleotide sequence of CP region of ToLCNDV (TN MET BoG2 and TN NGK BoG1) with other Begomovirus isolates. The tree was generated using the Neighbour-joining method in MEGA 6. A bootstrap analysis with 1000 replicates was performed and bootstrap percent values more than 70 are numbered along branches.

al. (2011) reported that, mosaic and puckering of leaves on summer squash were caused by the infection of SLCCNV in India. Sohrab et al. (2010) described the association of ToLCNDV with chlorosis and curly stunt disease of bottle gourd in North India. In the present study, preliminary screening with Deng540/541 universal begomovirus primer pair, as described by Deng et al. (1994), showed positive for the begomovirus association of with the bottle gourd samples of Tamil Nadu. Saha et al. (2014) also detected the ToLCNDV association with Tomato in North India with the same set of primers. Zepeda et al. (2007) used the complete coat protein (CP) gene nucleotide sequence for provisional identification of species in *Begomovirus*. In order to identify and to study the phylogenetic relationships of the *Begomovirus* associated with the bottle gourd of Tamil Nadu, complete coat protein gene was amplified using GKToLCV F/R primer pair (Nagendran et al. 2014a).

Coat protein sequence of TN MET BoG2 isolate shared maximum identity at both nucleotide and protein level with the Spain isolates of ToLCNDV reported on cucurbitaceous crops than ToLCNDV reported from Tomato. Also TN NGK BoG 1 isolate is showing highest identity towards isolates from Asian countries like India,

Table 1. Percentage identity of nucleotide (Nt) and amino acid (aa) sequences of CP gene encoded by ToLCNDV (TN MET BoG2 and TN NGK BoG1) with other selected *Begomovirus* for the study

Accession	Virus	TN MET BoG2		TN NGK BoG1	
		Nt	aa	Nt	aa
FN645905	*Tomato leaf curl New Delhi virus* clone 4-RCA-AI-F	92.8	95.7	94.8	98.4
AM747291	*Tomato leaf curl New Delhi virus* - Bitter Gourd isolate Mn-05	92.8	95.7	94.2	98.4
AM491590	*Bitter gourd yellow vein virus*-[Pakistan:Lahore:2004] clone BGAL4(1)	91.9	94.9	93.6	97.6
AY939926	*Tomato leaf curl New Delhi virus*	93.3	95.7	95.2	98.4
HM989845	*Tomato leaf curl New Delhi virus*-JLX10	92.9	95.7	94.6	98.4
KC513822	*Tomato leaf curl New Delhi virus*-India isolate Lucknow	93.5	96.1	94.2	98.8
HM007120	*Tomato leaf curl New Delhi virus*-India [India/Tumkur/Chilli/2008] clone pChTumB2	93.5	95.7	95.0	98.4
AM286434	*Tomato leaf curl New Delhi virus*-[Pumpkin:New Delhi] isolate 2	93.2	95.7	95.2	98.4
JN208136	*Tomato leaf curl New Delhi virus* isolate India:Ash gourd:2011	92.7	94.1	94.6	96.8
AF102276|	*Tomato leaf curl New Delhi virus*-[Luffa]	92.4	94.9	93.9	97.6
KF571461	*Tomato leaf curl New Delhi virus* isolate Tom5	92.8	95.3	96.2	98.0
KJ778692	*Tomato leaf curl New Delhi virus* isolate X1A [Iran:Eggplant]	91.8	95.3	98.4	99.6
KF002409	*Tomato leaf curl New Delhi virus* isolate parthenium [Pakistan]	91.8	95.3	98.4	99.6
HQ264185	*Tomato leaf curl New Delhi virus* isolate eggplant [India: Maharastra]	91.8	95.3	98.4	99.6
KF749223	*Tomato leaf curl New Delhi virus* isolate Almeria 661	93.9	97.2	91.1	97.6
KF749225	*Tomato leaf curl New Delhi virus* isolate Murcia 11.1	93.9	97.2	91.1	97.6
KF891468	*Tomato leaf curl New Delhi virus* isolate ToLCNDV-Spain-Almeria	93.9	97.2	91.1	97.6
KF551576	*Tomato leaf curl New Delhi virus* isolate TC309	93.9	96.4	94.6	98.4
EU487031	*Squash leaf curl China virus* isolate P54	88.0	92.9	89.3	95.3
EU543562	*Squash leaf curl China virus*-[Wax Gourd:Nakhon Pathom]	87.6	90.2	88.7	92.2
KC171648	*Squash leaf curl China virus* isolate GZ01	88.0	93.3	89.7	95.7
AM260206	*Squash leaf curl China virus* isolate G25	88.0	94.1	89.3	96.4
AB027465	*Squash leaf curl China virus*	87.4	92.9	88.8	95.3
AJ132711	*Tomato yellow leaf curl virus*-isolate Iran	72.0	74.9	71.8	75.6
HM625838	*Tomato leaf curl Gujarat virus*-[Pune:2008] clone JGB2	74.4	77.4	73.5	78.5
KC456161	*Tomato leaf curl Palampur virus* isolate Punjab	83.7	89.1	83.5	89.8
KF663698	*Tomato leaf curl Bangalore virus* isolate TC265	80.1	86.3	80.5	88.3
KM 275606	*Tomato leaf curl New Delhi virus* isolate TN MET BoG2	100	100	91.1	94.9
KM275616	*Tomato leaf curl New Delhi virus* isolate TN NGK BoG1	91.1	94.9	100	100

Iran and Pakistan. In phylogenetic analysis, TN NGK BoG1 isolates is closer with the ToLCNDV reported from parthenium and Brinjal. This shows virus infecting bottle gourd and *Parthenium* are same and the parthenium is acting as a reservoir host to this virus when the bottle gourd is not available. There are several evidences for the weeds acting as reservoir for the ToLCNDV infecting crop plants demonstrated by Haider *et al.,* (2006) on *Eclipta prostrata* and Reddy *et al.,* (2005) on *Croton bonplandianum*. These weeds are playing a mojor role in dissemination of virus to cultivated crops. In phylogenetic analysis, TN MET BoG2 and TN NGK BoG 1 were clustered along with the ToLCNDV rather than with SLCCNV, TYLCV (*Tomato yellow leaf curl virus*), ToLCPMV (*Tomato leaf curl Palampur virus*) and ToLCGV (*Tomato leaf curl Gujarat virus*). Several viruses like *Tomato leaf curl New Delhi virus* (Sohrab et al., 2010), *Papaya ring spot virus-W* (Mantri et al. 2004), *Zucchini yellow mosaic virus* (Verma et al. 2004) have been reported earlier on bottle gourd from India. Our study confirms the occurrence of ToLCNDV on bottle gourd in Tamil Nadu for the first time.

From this study it is inferred that, more than one kind of symptoms are produced by the infection of single virus. So it is very difficult to identify a virus based on symptomatology. Also, weeds are acting as a reservoir for the dissemination of viruses to cultivated crop plants, precise detection of virus through molecular techniques is necessary for the development of the better management strategies.

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सारांश

लौकी के पौधे पर रोग लक्षण प्रदर्शित मोजैक चित्ती, पर्णहिनता एवं पत्तियों का पीला पड़ना को तमिलनाडु के दो स्थानों में विगोमोवायरस का संक्रमण यूनिवर्सल प्राइमर पेयर पी सी आर विश्लेषण से स्पष्ट हुआ। बी एल ए एस टी विश्लेषण से इसकी पहचान टोमैटो लीफ कर्ल नई दिल्ली (टी ओ एल सी एन डी वी) के रूप में हुई। संयोजन रूप में दूसरे प्राइमर पेयर जी के टी ओ एल सी वी एफ/आर से पूर्ण कोट प्रोटीन जीन को एम्पलीफाई किया गया और इसे जीन बैंक डाटाबेस (टी एन एम इ टी बी ओ जी–2 के एम 275606, टी एन एन जी के बी ओ जी आई– के एम 275616) में जमा किया गया। टी एन एम टी बी ओ जी– 2 तथा टी एन एन जी के बी ओ जी–1 में न्यूक्लियोटाइड की अधिकतम एकात्मता 94 प्रतिशत तथा 98 प्रतिशत टी ओ एल सी एन डी वी के प्रति स्पेन व एशियन देशों से क्रमशः सूचित किया गया है।

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